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Skeletal Morphogenesis and Embryonic Development

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Formation of the skeletal system is one of the hallmarks that distinguish vertebrates from invertebrates. In higher vertebrates (i.e., birds and mammals), the skeletal system contains mainly cartilage and bone, which are mesoderm-derived tissues formed by chondrocytes and osteoblasts, respectively, during embryogenesis. A common mesenchymal progenitor cell, also referred as the osteochondral progenitor, gives rise to both chondrocytes and osteoblasts. The first overt sign of skeletal development is the formation of mesenchymal condensations, in which mesenchymal progenitor cells aggregate at future skeletal locations. Mesenchymal cells in different parts of the embryo come from different cell lineages. Neural crest cells give rise to craniofacial bones, the sclerotome compartment of the somites gives rise to most axial skeletal elements, and lateral plate mesoderm forms the limb mesenchyme, from which limb skeletons are derived (Fig. 1.1). Skeletal formation proceeds through two major mechanisms: intramembranous and endochondral ossification. In intramembranous ossification, osteochondral progenitors differentiate directly into osteoblasts to form membranous bone; during endochondral ossification, osteochondral progenitors differentiate into chondrocytes to form a cartilage template of the future bone. The location of each skeletal element determines its ossification mechanism and anatomic properties such as shape and size. This positional identity is acquired early in embryonic development, before mesenchymal condensation, through a process called pattern formation.

Cell–cell communication plays a critical role in pattern formation, and is mediated by several major signaling pathways. These include Wnts, Hedgehogs (Hhs), bone morphogenetic proteins (Bmps), fibroblast growth factors (Fgfs), and Notch/Delta. These pathways are also used later in skeletal development to control cell fate determination, proliferation, maturation, and polarity.

EARLY SKELETAL PATTERNING

Craniofacial patterning

Neural crest cells are the major source of cells establishing the craniofacial skeleton [1]. Reciprocal signaling between and among neural crest cells and epithelial cells (surface ectoderm, neural ectoderm or endodermal cells) ultimately establishes the identities of craniofacial skeletal elements [2].

Axial patterning

The most striking feature of axial skeletal patterning is the periodic organization of the vertebral column into multiple vertebrae along the anterior–posterior (A–P) axis. This pattern is established when somites, which are segmented mesodermal structures located on either side

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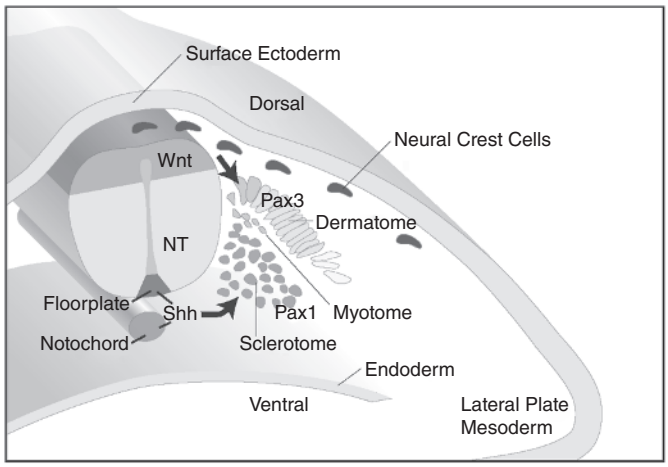

Fig. 1.1. Cell lineage contribution of chondrocytes and osteoblasts. Neural crest cells are born at the junction of dorsal neural tube and surface ectoderm. In the craniofacial region, neural crest cells from the branchial arches differentiate into chondrocytes and osteoblasts. In the trunk, axial skeletal cells are derived from the ventral somite compartment, sclerotome. Shh secreted from the notochord and floor plate of the neural tube induces the formation of sclerotome, which expresses Pax1. Wnts produced in the dorsal neural tube inhibit sclerotome formation and induce the dermomyotome, which expresses Pax3. Cells from the lateral plate mesoderm will form the limb mesenchyme, from which limb skeletons are derived.

of the neural tube, bud off at a defined pace from the anterior tip of the presomitic mesoderm (PSM) [3]. Somites give rise to the axial skeleton, striated muscle, and dorsal dermis [4–7]. The patterning of the axial skeleton is controlled by a molecular oscillator, or segmentation clock, that acts in the PSM [Fig. 1.2(A)]. The segmentation clock is operated by a traveling wave of gene expression (or cyclic gene expression) along the embryonic A–P axis, which is generated by an integrated network of the Notch, Wnt/β-catenin and fibroblast growth factor (FGF) signaling pathways [Fig. 1.2(B)] [8, 9].

The Notch signaling pathway mediates short-range communication between contacting cells [10]. The majority of cyclically expressed genes in the segmentation clock are targets of the Notch signaling pathway. The Wnt/β-catenin and FGF pathways mediate long-range signaling across several cell diameters. Upon activation of the Wnt pathway, β-catenin is stabilized and translocates to the nucleus where it activates the expression of downstream genes that are rhythmically expressed in the PSM [9, 11–13]. FGF signaling is also activated periodically in the posterior PSM [14, 15]. There is extensive cross-talk among these major oscillating signaling pathways; it is likely that each of the three pathways has the capacity to generate its own oscillations, while interactions among them allow efficient coupling and entrainment [16, 17]. Retinoic acid (RA) signaling controls somitogenesis by regulating the competence of PSM cells to undergo segmentation via antagonizing FGF signaling [Fig. 1.2(A)] [18, 19].

The functional significance of the segmentation clock in human skeletal development is highlighted by congenital axial skeletal diseases. For instance, mutations in Notch signaling components cause at least two human disorders, spondylocostal dysostosis (SCD, #277300, #608681, and #609813) and Alagille syndrome (AGS, OMIM# 118450 and #610205), both of which include vertebral column segmentation defects.

Once formed by the segmentation mechanism described above, somites are patterned along the dorsal–ventral axis by secreted signals derived from the surface ectoderm, neural tube and notochord (Fig. 1.1). The sclerotome forms from the ventral region of the somite, and gives rise to the axial skeleton and the ribs. Sonic hedgehog (Shh) from the notochord and ventral neural tube is required to induce sclerotome formation [20, 21] (Fig. 1.1) [22, 23]. In mice that lack *Shh*, the vertebral column and posterior ribs fail to form [24].

Limb patterning

Limb skeletons are patterned along the proximal–distal (P–D, shoulder to digit tip), anterior–posterior (A–P, thumb to little finger), and dorsal–ventral (D–V, back of the hand to palm) axes (Fig. 1.3). Along the P–D axis, the limb skeletons form three major segments: humerus or femur at the proximal end, radius and ulna or tibia and fibula in the middle, and carpal/tarsal, metacarpal/metatarsal, and digits in the distal end. Along the A–P axis, the radius and ulna have distinct morphological features; so do each of the five digits. Skeletal elements are also patterned along the D–V limb axis. For instance, the sesamoid processes are located ventrally whereas the patella forms on the dorsal side of the knee. Limb patterning events are regulated by three signaling centers in the early limb primodium, known as the limb bud, that act prior to mesenchymal condensation.

The apical ectoderm ridge (AER), a thickened epithelial structure formed at the distal tip of the limb bud, is the signaling center that directs P–D limb outgrowth (Fig. 1.3). Canonical Wnt signaling activated by Wnt3 induces AER formation [25], whereas BMP signaling leads to AER regression to halt limb extension [26]. Multiple FGF family members are expressed in the AER, but Fgf8 alone is sufficient to mediate the function of AER [27–29]. Fgf10 is expressed in the presumptive limb mesoderm and is required for initiation of limb bud formation; it subsequently controls limb outgrowth by maintaining *Fgf8* expression in the AER [30–32].

The second signaling center is the zone of polarizing activity (ZPA), a group of mesenchymal cells located at the posterior distal margin of the limb bud, immediately adjacent to the AER [Fig. 1.3(B)]. The ZPA patterns digit identity along the A–P axis. When ZPA tissue is grafted to a host limb bud on the anterior side under the AER, it leads to digit duplications in a mirror image of the

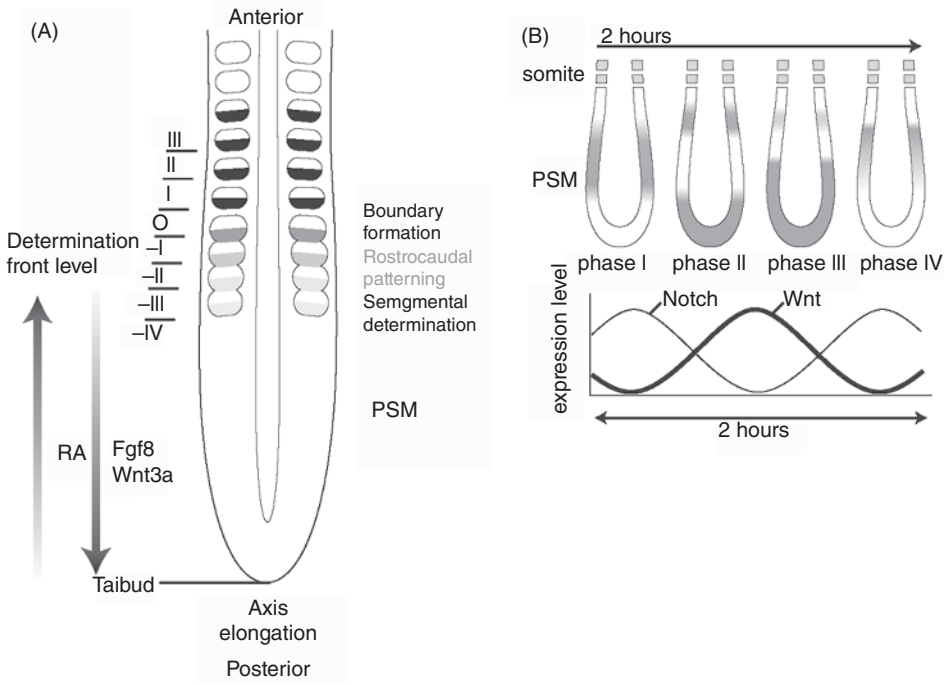

Fig. 1.2. Periodic and left–right symmetrical somite formation is controlled by signaling gradients and oscillations. (A) Somites form from the presomitic mesoderm (PSM) on either side of the neural tube in an anterior to posterior (A–P) wave. Each segment of the somite is also patterned along the A–P axis. Retinoic acid signaling controls the synchronization of somite formation on the left and right side of the neural tube. The most recent visible somite is marked by "0," whereas the region in the anterior PSM that is already determined to form somites is marked by a determination front that is determined by Fgf8 and Wnt3a gradients. This FGF signaling gradient is antagonized by an opposing gradient of retinoic acid. (B) Periodic somite formation (one pair of somite/2 hours) is controlled by a segmentation clock, the molecular nature of which is oscillated expression of signaling components in the Notch and Wnt pathways. Notch signaling oscillates out of phase with Wnt signaling.

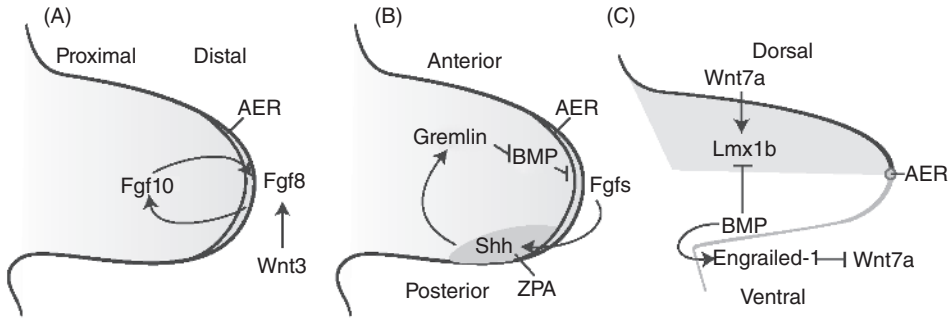

Fig. 1.3. Limb patterning and growth along the proximal–distal (P–D), anterior–posterior (A–P) and dorsal–ventral (D–V) axes are controlled by signaling interactions and feedback loops. (A) A signaling feedback loop between Fgf10 in the limb mesoderm and Fgf8 in the AER is required to direct P–D limb outgrowth. Wnt3 is required for AER formation. (B) Shh in the ZPA controls A–P limb patterning. A–P and P–D limb patterning and growth are also coordinated through a feedback loop between Shh and Fgfs expressed in the AER. Fgf signaling from the AER is required for Shh expression. Shh also maintains AER integrity by regulating Gremlin expression. Gremlin is a secreted antagonist of BMP signaling that promotes AER degeneration. (C) D–V patterning of the limb is determined by Wnt7a and BMP signaling through regulating the expression of Lmx1b in the limb mesenchyme.

endogenous ones [33]. *Shh* is expressed in the ZPA and is necessary and sufficient to mediate ZPA activity [34, 35]. However, the A–P axis of the limb is established prior to Shh signaling. This pre-Shh A–P limb patterning is controlled by combined activities of multiple transcription factors, including Gli3, Alx4, and the basic helix-loop-helix (bHLH) transcription factors dHand and Twist1. Mutations in the human *TWIST1* gene cause Saethre-Chotzen syndrome (SCS, OMIM#101400). The hallmarks of this syndrome are premature fusion of the calvarial bones and limb abnormalities. Mutations in the *GLI3* gene cause Greig cephalopolysyndactyly syndrome (GCPS, OMIM#175700) and Pallister-Hall syndrome (PHS, OMIM#146510), which are characterized by limb malformations.

The third signaling center is the non-AER limb ectoderm that covers the limb bud. This tissue controls D–V polarity of the ectoderm itself and also of the underlying mesoderm [Fig. 1.3(C)] (reviewed in Refs. 36 and 37). Wnt and BMP signaling control D–V limb polarity. *Wnt7a* is expressed in the dorsal limb ectoderm and activates the expression of *Lmx1b*, which encodes a dorsal-specific LIM homeobox transcription factor that determines the dorsal identity [38, 39]. *Wnt7a* expression is suppressed by the transcription factor En-1 in the ventral ectoderm [40]. The BMP signaling pathway is also ventralizing in the early limb [Fig. 1.3(C)]. The effects of BMP signaling in this ventralization are mediated by the transcription factors Msx1 and Msx2. The function of BMP signaling in the early limb ectoderm is upstream of En-1 in controlling D-V limb polarity [41]. However, BMPs also have En-1-independent ventralization activity by directly signaling to the limb mesenchyme to inhibit *Lmx1b* expression [42].

Limb development is a coordinated three-dimensional event. Indeed, the three signaling centers interact with each other through interactions of the mediating signaling molecules. First, there is a positive feedback loop between Shh expressed in the ZPA to maintain expression of FGFs in the AER, which connects A–P limb patterning with P–D limb outgrowth [Fig. 1.3(B)] [43–45]. This positive feedback look is antagonized by an FGF/Grem1 inhibitory loop that attenuates FGF signaling and thereby terminates limb outgrowth in order to maintain a proper limb size [46]. Second, the dorsalizing signal Wnt7a is also required for maintaining the expression of Shh that patterns the A–P axis [47, 48]. Third, Wnt/β-catenin signaling is both distalizing and dorsalizing [49–51].

EMBRYONIC CARTILAGE AND BONE FORMATION

The early patterning events described above determine where and when mesenchymal cells condense. Subsequently, the osteochondral progenitors in these condensations must form either chondrocytes or osteoblasts. Sox9 and Runx2 are master transcription factors that are required for the determination of chondrocyte and osteoblast cell fates, respectively [52–55]. Both are expressed in the osteochondral progenitor cells that constitute the mesenchymal condensations in the limb. *Sox9* expression precedes that of *Runx2* [56]. Coexpression of Sox9 and Runx2 in osteochondral progenitors is terminated when Sox9 and Runx2 expression is segregated into differentiated chondrocytes and osteoblasts, respectively [57]. The requirement for Runx2 in bone formation was demonstrated by the finding that *Runx2*$^{-/-}$ mice have no differentiated osteoblasts [52, 53]. Humans carrying heterozygous null mutations of the *RUNX2* gene have cleidocranial dysplasia (CCD, OMIM#119600), an autosomal-dominant condition characterized by hypoplasia/aplasia of clavicles, patent fontanelles, supernumerary teeth, short stature, and other changes in skeletal patterning and growth [53].

A number of transcriptional regulators that interact with Runx2 to control osteoblast differentiation have been identified. Zfp521 regulates osteoblast differentiation by HDAC3-dependent attenuation of Runx2 activity [58]. In addition, Runx2 mediates the function of Notch signaling in regulating osteoblast differentiation [59, 60].

Signaling through the Wnt and Indian hedgehog (Ihh) pathways is required for cell fate determination of osteoprogenitors into chondrocytes or osteoblasts by controlling the expression of *Sox9* and *Runx2*. Enhanced Wnt/β-catenin signaling increased bone formation and *Runx2* expression, but inhibited chondrocyte differentiation and *Sox9* expression [61–63]. Conversely, blocking Wnt/β-catenin signaling by removing β-*catenin* or *Lrp5* and *Lrp6* in osteochondral progenitor cells resulted in ectopic chondrocyte differentiation at the expense of osteoblasts [63–66]. Therefore, Wnt/β-catenin signaling levels in the condensation determine the outcome of bone formation. Relatively high Wnt/β-catenin signaling in intramembranous ossification allows direct osteoblast differentiation in the condensation, whereas during endochondral ossification, Wnt/β-catenin signaling in the condensation is initially lower, such that only chondrocytes differentiate. At later stages of endochondral ossification, Wnt/β-catenin signaling is upregulated at the periphery of the cartilage, driving osteoblast differentiation.

Ihh signaling is required for osteoblast differentiation only during endochondral bone formation by activating Runx2 expression [67, 68]. When Ihh signaling is inactivated in perichondrial cells, they ectopically form chondrocytes that express Sox9 at the expense of Runx2. Genetic epistatic tests showed that that β-*catenin* is required downstream of *Ihh* to promote osteoblast maturation [69]. In accordance, Ihh signaling is not (required once osteoblasts express osterix *Osx*) [70], a maker for cells committed to the osteoblast fate [71].

BMPs are transforming growth factor β (TGFβ) superfamily members that were identified as secreted proteins able to promote ectopic cartilage and bone formation [72]. Unlike Ihh and Wnt signaling, BMP signaling pro-

motes the differentiation of both osteoblasts and chondrocytes from mesenchymal progenitors. Reducing BMP signaling by removing BMP receptors leads to impaired chondrocyte and osteoblast differentiation and maturation [73]. The mechanisms underlying this unique property of BMPs have been under intense investigation for the past two decades. Our understanding of BMP action in chondrogenesis and osteogenesis has benefited greatly from molecular studies of BMP signal transduction [74].

The functions of FGF pathways in mesenchymal condensation and osteochondral progenitor differentiation remain to be elucidated, as complete genetic inactivation of FGF signaling in mesenchymal condensations has not been achieved. Nevertheless, it is clear that FGFs act in mesenchymal condensations to control intramembranous bone formation. FGF signaling can promote or inhibit osteoblast proliferation and differentiation depending on the cell context. Mutations in the genes encoding FGFR 1, 2, and 3 cause craniosynostosis (premature fusion of the cranial sutures). All of these mutations are autosomal dominant and many of them are activating mutations. The craniosynostosis syndromes involving FGFR1, 2, 3 include Apert syndrome (AS, OMIM# 101200), Beare-Stevenson cutis gyrata (OMIM#123790), Crouzon syndrome (CS, OMIM#123500), Pfeiffer syndrome (PS, OMIM#101600), Jackson-Weiss syndrome (JWS, OMIM#123150), Muenke syndrome (MS, OMIM#602849), crouzonodermoskeletal syndrome (OMIM#134934), and osteoglophonic dysplasia (OGD, OMIM#166250).

CHONDROCYTE PROLIFERATION AND DIFFERENTIATION IN THE DEVELOPING CARTILAGE

During endochondral bone formation, chondrocytes differentiate from osteochondral progenitor cells to form cartilage, which provides a growth template for the future bone. Chondrocytes undergo a tightly controlled program of progressive proliferation and hypertrophy, which is required for endochondral bone formation. In the developing cartilage of the long bone, chondrocytes at different stages of differentiation are located in distinct zones along the longitudinal axis and such organization is required for long bone elongation [Fig. 1.4(A)]. Proliferating chondrocytes express *Col2a1* (ColII), whereas hypertrophic chondrocytes express *Col10a1* (ColX). The chondrocytes that have exited the cell cycle, but have not yet become hypertrophic, are known as prehypertrophic chondrocytes. Chondrocytes either remain in one zone (i.e., those in the permanent cartilage) or transit to other zones in order (i.e., those in the growth plate) during development or homeostasis. This progression is precisely regulated by multiple signaling pathways.

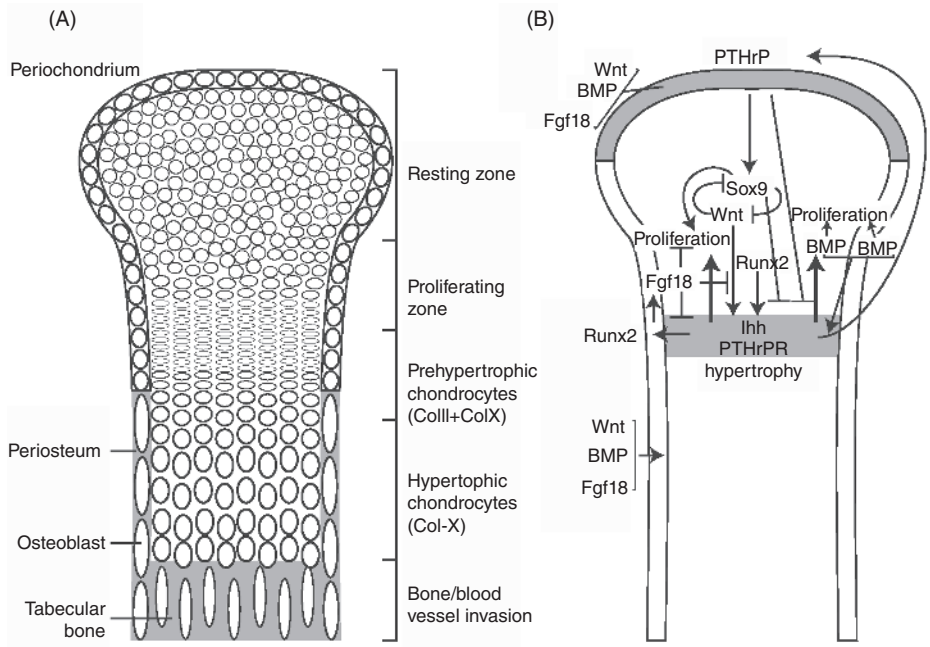

Fig. 1.4. Chondrocyte proliferation and hypertrophy are tightly controlled by signaling pathways and transcription factors. (A) Schematic drawing of a developing long bone cartilage. Chondrocytes with different properties of proliferation have different morphologies and are located in distinct locations along the longitudinal axis. See text for details. (B) Molecular regulation of chondrocyte proliferation and hypertrophy. Ihh, PTHrP, Wnt, FGF, and BMP are major signaling pathways that control chondrocyte proliferation and hypertrophy. A negative feedback loop between Ihh and PTHrP is fundamental in regulating the pace of chondrocyte hypertrophy. Transcription factors Sox9 and Runx2 act inside the cell to integrate signals from different pathways. See text for details.

Ihh is expressed in prehypertrophic and early hypertrophic chondrocytes and acts as a master regulator of endochondral bone development by promoting chondrocyte proliferation, controlling the pace of chondrocyte hypertrophy and coupling cartilage development with bone formation by inducing osteoblast differentiation in the adjacent perichondrium [67].

Ihh$^{-/-}$ mice have striking skeletal defects, including a lack of endochondral bone formation and smaller cartilage elements due to a marked decrease in chondrocyte proliferation and acceleration of hypertrophy [67, 75]. Ihh controls the pace of chondrocyte hypertrophy by activating the expression of parathyroid hormone related peptide (*PTHrP*) in articular cartilage and periarticular cells [67, 76]. PTHrP acts on the same G-protein-coupled receptors used by parathyroid hormone (PTH). These PTH/PTHrP receptors (*PPR*s) are expressed at high levels by prehypertrophic and early hypertrophic chondrocytes. PTHrP signaling is required to inhibit precocious chondrocyte hypertrophy primarily by keeping proliferating chondrocytes in the proliferating pool [77, 78]. Ihh and PTHrP form a negative feedback loop to control the chondrocyte's decision whether or not to leave the proliferating pool and become hypertrophic [Fig. 1.4(B)]. In this model, PTHrP, secreted from cells at the ends of cartilage, acts on proliferating chondrocytes to keep them proliferating. When chondrocytes displaced far enough from the source of PTHrP that the PPRs are no longer activated, they exit the cell cycle and become Ihh-producing prehypertrophic chondrocytes. Ihh diffuses through the growth plate to stimulate PTHrP expression at the ends of cartilage as way to slow down hypertrophy. This model is supported by experiments using chimeric mouse embryos [79]. Clones of *PPR*$^{-/-}$ chondrocytes differentiate into hypertrophic chondrocytes and produce Ihh within the wild type proliferating chondrocyte domain. This ectopic Ihh expression leads to ectopic osteoblast differentiation in the perichondium, upregulation of PTHrP expression, and a consequent lengthening of the columns of wild type proliferating chondrocytes. These studies demonstrate that the lengths of proliferating columns, and hence the elongation potential of cartilages, are critically determined by the Ihh–PTHrP negative feedback loop. Indeed, mutations in *IHH* in humans cause brachydactyly Type A1 (OMIM#112500), which is characterized by shortened digit phalanges and short body statue [80].

Several Wnt ligands are expressed in the cartilage and perichondrium of mouse embryos [62, 81]. Some activate canonical (β-catenin-dependent) and others activate noncanonical (β-catenin-independent) pathways to regulate chondrocyte proliferation and hypertrophy. In the absence of either canonical or noncanonical Wnt signaling, chondrocyte proliferation is altered and hypertrophy is delayed [63, 81, 82]. Furthermore, both canonical and noncanonical Wnt pathways act in parallel with Ihh signaling to regulate chondrocyte proliferation and differentiation [69, 81]. Wnt and Ihh signaling may regulate common downstream targets such as Sox9 (see below) [81, 82].

Many FGF ligands and receptors (FGFRs) are expressed in the developing cartilage. The significant role of FGF signaling in skeletal development was first realized by the discovery that achondroplasia (ACH, OMIM#100800), the most common form of skeletal dwarfism in humans, was caused by a missense mutation in *FGFR3*. Later, hypochondroplasia (HCH, OMIM#146000), a milder form of dwarfism, and thanatophoric dysplasia (TD, OMIM# 187600 & 187601), a more severe form of dwarfism, were also found to result from mutations in *FGFR3*. Signaling through FGFR3 negatively regulates chondrocyte proliferation and hypertrophy [83–90], in part by direct signaling in chondrocytes [83, 84] to activate Janus kinase–signal transducer and activator of transcription-1 (Jak–Stat1) and the MAPK pathways [85]. FGFR3 signaling also interacts with the Ihh/PTHrP/BMP signaling pathways [86, 87].

Since *Fgf18*$^{-/-}$ mice exhibit a phenotype including increased chondrocyte proliferation that closely resembles the cartilage phenotypes of *Fgfr3*$^{-/-}$ mice, Fgf18 is likely a physiological ligand for FGFR3 in the mouse. However, the phenotype of the *Fgf18*$^{-/-}$ mouse is more severe than that of the *Fgfr3*$^{-/-}$ mice, suggesting that Fgf18 signals through FGFR1 in hypertrophic chondrocytes and through FGFR2 and -1 in the perichondrium. Mice conditionally lacking FGFR2 develop skeletal dwarfism with decreased bone mineral density [88, 89)]. Osteoblasts also express FGFR3, and mice lacking *Fgfr3* are osteopenic [90, 91]. Thus in osteoblasts, FGF signaling positively regulates bone growth by promoting osteoblast proliferation. Interestingly, mice lacking *Fgf2* also show osteopenia, though much later in development than in *Fgfr2*-deficient mice [92], suggesting that Fgf2 may be a homeostatic factor that replaces the developmental growth factor, Fgf18, in adult bones. It is still not clear which FGFR responds to Fgf2/18 in osteoblasts.

Like the other major signaling pathways mentioned above, BMP signaling also acts during later stages of cartilage development. Both *in vitro* explant experiments and *in vivo* genetic studies showed that BMP signaling promotes chondrocyte proliferation and *Ihh* expression. The addition of BMPs to limb explants increases proliferation of chondrocytes whereas Noggin blocks chondrocyte proliferation [86, 93]. In addition, conditional removal of both *BmpRIA* and *BmpRIB* in differentiated chondrocytes leads to reduced chondrocyte proliferation and Ihh expression. BMP signaling also regulates chondrocyte hypertrophy, as removal of *BmpRIA* in chondrocytes leads to an expanded hypertrophic zone due to accelerated chondrocyte hypertrophy and delayed terminal maturation of hypertrophic chondrocytes [94]. BMP signaling regulates chondrocyte proliferation and hypertrophy at least in part through regulating Ihh expression.

BMP and FGF signaling pathways are mutually antagonistic in cartilage [86]. Comparison of cartilage phenotypes of BMP and FGF signaling mutants indicate that these two signaling pathways antagonize each other in regulating chondrocyte proliferation and hypertrophy [94)].

The above signaling pathways mediate the majority of their effects on cell proliferation, differentiation, and survival by regulating the expression of key transcription factors. Sox9 and Runx2 are two critical transcription factors that integrate inputs from these signaling pathways. When *Sox9* was removed from differentiated chondrocytes, chondrocyte proliferation and the expression of matrix genes and the Ihh–PTHrP signaling components were reduced in the cartilage [56]. This phenotype is very similar to that of mice lacking both *Sox5* and *Sox6*, two other Sox-family members that require Sox9 for expression. Sox5 and Sox6 cooperate with Sox9 to maintain the chondrocyte phenotype to regulate chondrocyte specific gene expression [95]. Haploinsufficiency for *SOX9* in humans causes campomelic dysplasia (CD, OMIM# 114290), a condition that is recapitulated in $Sox9^{+/-}$ mice, and which includes cartilage hypoplasia and a perinatal lethal osteochondrodysplasia [96]. Chondrocyte hypertrophy is accelerated in the $Sox9^{+/-}$ cartilage, but delayed in *Sox9*-overexpressing cartilage [82, 96]. Sox9 acts in both the PTHrP and Wnt signaling pathways to control chondrocyte proliferation. PTHrP signaling in chondrocytes activates PKA, which promotes Sox9 transcriptional activity by phosphorylating it [97]. In addition, Sox9 inhibits Wnt/β–catenin signaling activity by promoting β–catenin degradation [82, 98]. Thus, Sox9 is a master transcription factor that acts in many critical stages of chondrocyte proliferation and differentiation as a central node inside prechondrocytes and chondrocytes to receive and integrate multiple signaling inputs.

In addition to its role in early osteoblast differentiation, Runx2 is expressed in prehypertrophic and hypertrophic chondrocytes and controls chondrocyte proliferation and hypertrophy. Chondrocyte hypertrophy is significantly delayed and Ihh expression is reduced in $Runx2^{-/-}$ mice, whereas *Runx2* overexpression in the cartilage results in accelerated chondrocyte hypertrophy [99, 100]. Furthermore, removing both *Runx2* and *Runx3* completely blocks chondrocyte hypertrophy and Ihh expression in mice, suggesting that Runx transcription factors control Ihh expression [101]. Thus, as with Sox9, Runx2 can be viewed as a master controlling transcription factor and a central node through which other signaling pathways are integrated in coordinate chondrocyte proliferation and hypertrophy. In chondrocytes, Runx2 acts in the Ihh-PThrP pathway to regulate cartilage growth by controlling the expression of Ihh. However, this cannot be its only function, as *Runx2* upregulation leads to accelerated chondrocyte hypertrophy, whereas *Ihh* upregulation leads to delayed chondrocyte hypertrophy. One of Runx2's Ihh-independent activities is to act in the perichondrium to inhibit chondrocyte proliferation and hypertrophy by regulating Fgf18 expression [102]. Interestingly, this role of Runx2 in the perichondrium is antagonistic to its role in chondrocytes. Recent studies have shown that histone deacetylase 4 (HDAC4), which governs chromatin structure and represses the activity of specific transcription factors, regulates chondrocyte hypertrophy and endochondral bone formation by inhibiting the activity of Runx2 [103]. Runx2 interacts with the Gli3 repressor form Gli3rep, which inhibits DNA binding by Runx2 [104]. Therefore, one mechanism whereby Hh signaling promotes osteoblast differentiation may be through enhancing Runx2 DNA binding by reducing the generation of Gli3rep.

Developing skeletal elements have distinct morphologies, which are required for their function. For example, the limb and the long bones preferentially elongate along the P–D axis. Although the molecular mechanism underlying such directional morphogenesis was poorly understood in the past, there is evidence that alignment of columnar chondrocytes in the growth plate is regulated by planar cell polarity (PCP) pathways during directional elongation of the cartilage [105, 106]. PCP is an evolutionarily conserved pathway that is required in many directional morphogenetic processes including left–right asymmetry, neural tube closure, body axis elongation and brain wiring [107–109]. Recently, a major breakthrough has been made by demonstrating that newly differentiated chondrocytes in developing long bones are polarized along the P–D axis. Vangl2 protein, a core regulatory component in the PCP pathway, is asymmetrically localized on the proximal side of chondrocytes [110]. The asymmetrical localization of Vangl2 requires a Wnt5a signaling gradient. In the $Wnt5a^{-/-}$ mutant limb, the cartilage forms a ball-like structure, and Vangl2 is symmetrically distributed on the cell membrane [110] (Fig. 1.5). Mutations in genes encoding PCP pathway components, such as *WNT5a* and *ROR2*, have been found in skeletal malformations such as the Robinow Syndrome and brachydactyly type B1, both of which are short-limb dwarfisms [111–115].

REGULATION OF CHONDROCYTE SURVIVAL

Apart from its proliferation, differentiation and polarity, chondrocyte survival is also highly regulated. The Wnt/β-catenin, Hh, and BMP pathways signaling are all important in chondrocyte survival. Chondrocyte cell death is significantly increased when β-catenin is removed [69]. Cartilage is also special as it is an avascular tissue that develops under hypoxia because chondrocytes, particularly those in the middle of the cartilage, do not have access to vascular oxygen delivery [116]. As in other hypoxic conditions, the transcription factor hypoxia-inducible factor 1 (Hif-1), and its oxygen-sensitive component Hif-1α, is the major mediator of the hypoxic response in developing cartilage. Removal of *Hif-1α* in cartilage results in chondrocyte cell death in the interior of the growth plate. A downstream target of Hif-1 in regulating the hypoxic response of chondrocytes is VEGF [117]. The extensive cell death seen in the cartilage of mice lacking *Vegfa* has a striking similarity to that observed in mice in which *Hif-1α* is removed in cartilage [116]. The Wnt/β-catenin, Hh, and BMP pathways signaling are all important in chondrocyte survival.

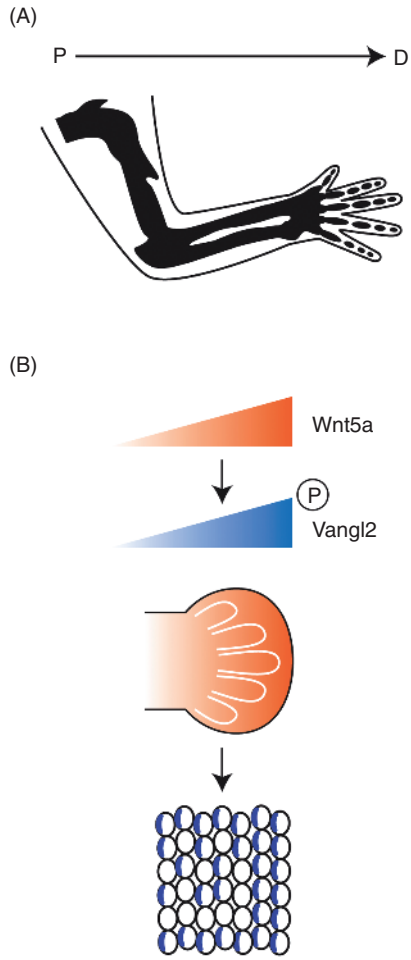

Fig. 1.5. Wnt5a gradient controls directional morphogenesis by regulating Vangl2 phosphorylation and asymmetrical localization. (A) Schematics of skeletons in a human limb that preferentially elongates along the proximal–distal axis. (B) A model of a Wnt5a gradient controlling P–D limb elongation by providing a global directional cue. Wnt5a is expressed in a gradient (orange) in the developing limb bud, and this Wnt5a gradient is translated into an activity gradient of Vangl2 by inducing different levels of Vangl2 phosphorylation (blue). In the distal limb bud of an E12.5 mouse embryo showing the forming digit cartilage, the Vangl2 activity gradient then induces asymmetrical Vangl2 localization (blue) and downstream polarized events.

Chondrocyte cell death is significantly increased when β-catenin is removed [69].

CONCLUSIONS

Skeletal formation is a process that has been perfected and highly conserved during vertebrate evolution. Understanding the molecular mechanisms regulating cartilage and bone formation during development will allow us to redeploy these pathways to promote skeletal tissue repair using endogenous cells, autologous cells and tissues, or iPS (induced pleuripotent stem) cells. Understanding skeletal development is also indispensable for understanding pathological mechanisms in skeletal diseases, finding therapeutic targets, promoting consistent cartilage or bone repair *in vivo* and eventually growing functional cartilage or bone *in vitro*.

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